



ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (BE), and starch debranching enzyme (DBE) (Smith *et al.*, 1997; Myers *et al.*, 2000; James *et al.*, 2003). Many starch biosynthetic enzymes are distributed between the soluble fraction of the plastids and the insoluble starch granules themselves (Ball and Morell, 2003; Tetlow *et al.*, 2004a).

The glucan biosynthetic system in plants is complex in the regard that multiple forms of each enzyme have been conserved in evolution and are co-expressed at the same site and time of starch accumulation. SSs catalyse the transfer of  $\alpha$ -D-glucose from the nucleotide sugar ADPGlc to the non-reducing end of a chain, forming a new  $\alpha$ -(1  $\rightarrow$ 4) linkage. In maize (*Zea mays* L.) five classes of SS have been identified, termed granule-bound starch synthase (GBSS), SSI, SSII, SSIII, and SSIV. Each of these SS classes is conserved in other plant species, and in cereals such as maize there are closely related isoforms in many of the classes (typically designated 'a' or 'b'). GBSS, which is localized exclusively within the starch granule, is responsible for amylose biosynthesis (Shure *et al.*, 1983) and also has a role in the elongation of long chains in amylopectin (Denyer *et al.*, 1996). Genetic analyses in *Arabidopsis* and rice suggest that SSI is required for the elongation of short linear chains within amylopectin having a degree of polymerization (DP) of approximately 6–7, to form chains of DP 8–12 (Delvalle *et al.*, 2005; Fujita *et al.*, 2006). For SSIIa, genetic analyses suggest its function is to elongate amylopectin chains of DP 6–10 to produce intermediate length chains of DP 12–25 (Zhang *et al.*, 2004). A second form of SSII, designated SSIIb, has also been identified by cDNA cloning (Harn *et al.*, 1998); however, the expression of this protein has not yet been demonstrated in cereal endosperm and its role remains to be defined. Analysis of SSIII mutants suggests this enzyme class catalyses the synthesis of long amylopectin chains of DP 25–35 or greater (Wang *et al.*, 1993; Gao *et al.*, 1998; Jane *et al.*, 1999). Although little is known about the role of SSIV in starch synthesis, recent research in *Arabidopsis* suggests this enzyme class may function in the control of granule number (Roldan *et al.*, 2007).

The role of the BEs in starch synthesis is to catalyse the formation of branch points within glucan chains by cleaving an  $\alpha$ -(1  $\rightarrow$ 4) linkage and transferring the released chain to another linear glucan via an  $\alpha$ -(1  $\rightarrow$ 6) linkage. Three BEs are present in maize and all are expressed in maize endosperm: BEI, BEIIa, and BEIIb (Boyer and Preiss, 1978; Fisher *et al.*, 1993; Guan and Preiss, 1993). Loss of BEIIb causes an altered form of amylopectin having fewer short chains and increased proportions of longer length chains (Boyer and Preiss, 1981; Fisher *et al.*, 1993). Mutations eliminating BEIIa expression caused slight alterations of leaf starch but had little effect on the structure of endosperm (Blauth *et al.*,

2001). Similarly, mutations in the gene encoding BEI have no notable effect on either leaf or endosperm starch (Blauth *et al.*, 2002).

Granule-associated proteins can be divided into two categories according to their degree of association with starch granules. One group consists of surface-associated proteins and the other comprises internal granule-associated proteins. Surface-associated proteins have a small degree of association with the starch granule and can be separated from the granule by protease digestion (with thermolysine, proteinase K) or by extensive washing in aqueous buffer containing a detergent and a reducing agent, at a temperature below the gelatinization temperature for starch (Denyer *et al.*, 1993; Rahman *et al.*, 1995; Boren *et al.*, 2004). By contrast, extraction of internal granule-associated proteins requires the gelatinization of the starch granule in SDS in the presence of a reducing agent (Tsai, 1974; Echt and Schwartz, 1981; Denyer *et al.*, 1993).

Studies in many plant species have shown that starch biosynthetic enzymes exist as both soluble proteins in the stroma and as internal granule-associated proteins (Table 1). Enzymes that display this property in maize endosperm include SSI (Mu *et al.*, 1994, 2001), SSIIa (Zhang *et al.*, 2004), and BEIIb (Mu-Forster *et al.*, 1996). For these enzymes, it is unclear whether the stromal form, the granule-bound form, or both, are physiologically significant for starch biosynthesis. Previous granule protein identifications have been made largely through immunoblot analyses using specific antibodies, so there may be other starch metabolizing proteins located within the granule that have not yet been identified. One objective

**Table 1.** Known internal starch granule-associated proteins

Species	Enzymes identified in starch granules	Reference
Potato	GBSS (60 kDa); SSII (92 kDa); R1 (160 kDa)	Edwards <i>et al.</i> , 1995; Ritte <i>et al.</i> , 2000
Maize	GBSS (60 kDa); SSI (76 kDa); SSIIa (86 kDa), BEIIb (85 kDa)	Mu-Forster <i>et al.</i> , 1996; Zhang <i>et al.</i> , 2004
Rice	GBSS (60 kDa); SSI (72 kDa); SSIIa (86 kDa), BEIIb (82 kDa)	Umemoto and Aoki, 2005
Pea	GBSS (60 kDa); SSII (77 kDa); BEII (100 kDa); BEI (114 kDa); R1 (160 kDa)	Denyer <i>et al.</i> , 1993; Ritte <i>et al.</i> , 2000
Barley	GBSS (60 kDa); SSI (71 kDa); SSIIa (87 kDa); BEIIb (93 kDa); BEIc (140 kDa)	Boren <i>et al.</i> , 2004; Peng <i>et al.</i> , 2000
Wheat	GBSS (60 kDa); SSI (SGP-3, 80 kDa); SSIIa (SGP-B1, 100 kDa); SSIIa (SGP-D1, 108 kDa); SSIIa (SGP-A1, 115 kDa); BEIIb (SGP-2, 92 kDa); BEIc (SGP-140, 140 kDa); BEIc (SGP-145, 145 kDa); BEIIa	Rahman <i>et al.</i> , 1995; Regina <i>et al.</i> , 2005

of the current study was to use non-biased proteomic technology to characterize more fully the complement of proteins located in the interior of starch granules, i.e. the granule proteome. Several proteins not previously known to be located within maize starch granules were identified.

A second objective of the study was to use the granule proteome analysis as a means of investigating functional interactions among starch biosynthetic enzymes. In the last decade, a large number of mutants have been identified in maize by classical and reverse genetic methods that have contributed to our understanding of the function of each specific starch biosynthetic enzyme. In this study, internal granule-associated proteins were analysed in such mutants affected in either GBSS, SSIIa, SSIII, or BEIIb, in order to seek for pleiotropic effects that could indicate interactions between the proteins. Several such instances were identified involving SSIII, BEIIa, BEIIb, and BEI. Finally, granule proteins were surveyed for the presence of phosphoryl groups in order to investigate further whether protein phosphorylation could be involved in the regulation of starch biosynthesis. At least three proteins, GBSS, BEIIb, and starch phosphorylase, were found to be phosphorylated in their internal granule-associated form.

## Materials and methods

### Plant materials

Maize plants (*Zea mays* L.) were field-grown in the summer at Iowa State University or in France by Limagrain Genetics. Kernels from self-pollinated ears were collected 16 days after pollination (dap) and used fresh for amyloplast preparation, or dried at maturity and used for starch granule preparation. Wild-type maize was the W64A inbred line and all mutant alleles were backcrossed into this genetic background. In addition, the *su2*<sup>-</sup> and *dull1*<sup>-</sup> mutations were analysed in plants in the F566 genetic background. The following mutant alleles were used: *ae-B1*, a 882 bp deletion in the gene coding for BEIIb that removes all of exon 9 (Genbank accession no. AF072725) (Fisher *et al.*, 1996; M Yandeu-Nelson and M Gultinan, personal communication); *dul-M3*, a *Mutator* insertion in the first exon of the gene coding for SSIII (MG James, unpublished results); *su2-1981*, an uncharacterized spontaneous mutation known to condition lack of SSII protein (Zhang *et al.*, 2004); *wx1-C*, originally obtained from the Maize Genetics Cooperation Stock Center (ID no. 923L).

### Isolation of starch granules and internal granule-associated proteins

Starch granules were isolated from mature maize kernels. Dried kernels were incubated for 15 h at 40 °C in 0.3% (w/v) Na metabisulphite and 85% (v/v) lactic acid pH 3.8 to avoid any protease activity during rehydration. Endosperm tissue was manually dissected from pericarp and embryo tissues. The endosperm was homogenized in starch extraction buffer (50 mM TRIS-HCl pH 7, 10% glycerol, 10 mM EDTA, and 1.25 mM DTT) at 4 °C. The homogenate was passed through a 100 µm filter and centrifuged at 15 000 g for 15 min at 4 °C. The viscous yellow layer on top of the starch was carefully removed. The starch pellet was washed twice with starch extraction buffer, twice with cold 95% ethanol, and

twice with acetone, and then dried under a slight stream of air. At this point the starch is considered crude because it still contains surface proteins.

To extract the internal, tightly-associated granule proteins, starch was washed 3× in water, 3× in gelatinization buffer (62.5 mM TRIS-HCl, pH 6.8, 2% SDS, and 5% β-mercaptoethanol), and 3× in 95% ethanol. The granule proteins were released by boiling the starch in gelatinization buffer for 5 min. The boiling step in SDS-buffer also serves to inactivate the protease (Gorg *et al.*, 2004). The starch paste was kept on ice then centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was collected and passed through a 0.25 µm filter before being mixed in 4 vols of cold (−20 °C) acetone for at least 1 h to precipitate the proteins, then centrifuged at 10 000 g for 30 min. The resulting pellet was washed twice with cold acetone and dried under a gentle stream of air. The air-dried pellet was solubilized in a buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT, and 0.2% (v/v) Triton-X100.

To ensure reproducibility between completely independent growth times, place of growth, genetic backgrounds, and starch isolations, internal granule-associated proteins were extracted from different mature maize kernels grown in France or in the US. Protein extractions and SDS-PAGE characterizations were performed at least in triplicate to ensure reproducibility. Regardless of the biological origin, the same genotype-specific protein profile was obtained for each mutant and the wild-type control.

Protein concentration was determined using a Bio-Rad Protein Assay (catalogue no. 500-0006). Proteins extracted in triplicate from the same starch batch similar quantities of protein. In the wild type, for example, the yields were 40, 47, and 45 µg protein per 100 mg starch (an average of 44 µg protein per 100 mg). For *su2* and *ae* mutant starches, the yields were essentially the same as the wild type (average values of 40 µg and 45 µg protein per 100 mg starch, respectively). For *dul* mutant starch, the average amount was slightly lower than the wild type (33 µg protein per 100 mg starch) and for *wx* starch the average amount is very low (6 µg protein per 100 mg of starch).

### Preparation of amyloplasts

Amyloplast extracts were prepared as follows by modification of a procedure previously used for wheat endosperm (Tetlow *et al.*, 2004b). Endosperm tissue extracted from wild-type kernels harvested 16 dap was suspended in ice-cold amyloplast extraction buffer (50 mM HEPES-KOH, pH 7.5, 0.8 M sorbitol, 1 mM EDTA, 1 mM KCl, and 2 mM MgCl<sub>2</sub>) and finely minced to homogeneity with a razor blade. The slurry was filtered through Miracloth (CalBiochem catalogue no. 475855) and layered over 3% Histodenz (Sigma catalogue no. D2158) in amyloplast extraction buffer in approximately equal volumes. The lysates were centrifuged at 100 g at 4 °C for 20 min and the supernatant was carefully decanted. The starch pellet, with a yellow ring of amyloplast fraction on the top, was suspended in ice-cold rupture buffer [100 mM Tricine-KOH, pH 7.8, 1 mM EDTA, 1 mM DTT, 5 mM MgCl<sub>2</sub>, and 1× plant protease inhibitor cocktail (Sigma catalogue no. P9599)]. The suspension was centrifuged at 5000 rpm at 4 °C for 5 min. The supernatant was collected and centrifuged again at 50 000 rpm at 4 °C for 30 min. The supernatant was collected as an enriched amyloplast lysate and was stored (−80 °C) prior to experimentation.

### SDS-PAGE and gel staining

In order to overcome differences that could be due to different extraction yields, proteins from the different genotypes were compared based on total mass applied to the gel. This allows a relative quantification of these proteins between different genotypes. A volume containing 15 µg protein was loaded on

a 7.5% polyacrylamide gel for each mutant, with the exception of starch granule proteins from the *wx<sup>-</sup>* mutant, for which 3 µg protein was loaded. SDS-PAGE was performed using the Laemmli buffer system and 35 mA constant current per gel in a Protean II xi cell (Bio-Rad). Gels were stained either by Coomassie Blue G250 (Sigma-Aldrich) or silver-stained according to published procedures (Blum *et al.*, 1987), or SYPRO Ruby stained according to the manufacturer's instructions (Bio-Rad catalogue no. 170-3126). Phosphorylated proteins in the gels were stained using a Pro-Q Diamond Phosphoprotein Gel Stain kit (Invitrogen catalogue no. P33300) according to the manufacturer's instructions.

### LC-MS/MS analysis

Protein bands were excised from a Coomassie Blue stained gel and subjected to in-gel proteolytic digestion as follows. Gels bands were washed with 100 µl of 25 mM  $\text{NH}_4\text{HCO}_3$ , followed by 100 µl of 50% acetonitrile in 25 mM  $\text{NH}_4\text{HCO}_3$ . Proteins were then reduced by incubation with 10 mM DTT (1 h at 57 °C) and alkylated with 100 µl of 55 mM iodoacetamide for 45 min at room temperature. Gels bands were further washed with 25 mM  $\text{NH}_4\text{HCO}_3$  and then dried in a vacuum centrifuge. The proteins were digested overnight at 37 °C by the addition of 10–20 µl trypsin (12.5 ng µl<sup>-1</sup> in 25 mM  $\text{NH}_4\text{HCO}_3$ ). The resulting peptide mixture was acidified by the addition of 1 µl of aqueous solution of 1% formic acid and stored at -20 °C until analysis.

Nanoscale capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses of the digested proteins were performed using a Switchos-Ultimate capillary LC system (LC Packings/Dionex, Amsterdam, The Netherlands), coupled with a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Micromass/Waters, Manchester, UK). Chromatographic separation was conducted on a reverse-phase capillary column (Pepmap C18, 75 µm i.d., 15 cm length, LC Packings) at a flow rate of 200 nl min<sup>-1</sup>. The elution gradient consisted of a linear increase from 2–50% acetonitrile over 50 min, followed by a rapid increase to 60% acetonitrile over 10 min. Mass data acquisitions were performed with Mass Linx software (Micromass/Waters, Manchester, UK) using the 'data dependent acquisition' mode. MS data were recorded for 1 s on the mass-to-charge (*m/z*) range of 400–1500, after which the most intense ions were selected and fragmented in the collision cell (MS/MS measurements) for 8 s; MS/MS data were recorded in the *m/z* range of 50–1500. Original MS data are shown as supplementary data in Fig. S1 at JXB online.

### Database searches

LC-MS/MS data were processed using the Protein Lynx software (Waters) and further searched against databanks using Mascot 2.2 (Matrix Science). Protein identification was achieved by searching the peptide masses and MS/MS spectra against Uniprot/Swiss-Prot and UniProt/TrEMBL databanks, or in the Plant Transcript Assemblies (TaGI) databank (*Zea mays* L: release 06/01/2007) (<http://plantta.tigr.org/index.shtml>). One missed trypsin cleavage and two dynamic modifications (methionine oxidation and methylation of Glu or Asp) were allowed per peptide. Mass accuracy was set to 150 ppm for parent ions and 0.3 Da for MS/MS fragments. Protein identification generated by single MS/MS spectra were considered valid when the MS/MS spectra displayed a wide series of intense fragments which could be assigned to major predicted fragments (i.e. b or y ions) of the proposed peptide sequence. Proteins were identified with a minimum of two MS/MS spectra matching the databank sequence, with individual ion scores of more than 39 for Uniprot/Swiss-Prot databank searches, 50 for UniProt/TrEMBL databank, and 42 for TaGI databank.

### Immunoblotting

Rabbit polyclonal serum against GBSS (termed αGBSS) was obtained by subcutaneous immunization with a specific synthetic peptide (CERVWGKTEEK) corresponding to residues 166–175 of the full-length sequence (GenBank accession no CAA27574). A cysteine residue was added to the N-terminus to allow covalent coupling to carrier proteins, BSA and KLH, via the thiol function using MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) (NeoMPS, Strasbourg). BSA-peptide conjugate (300 µg per injection) emulsified with Freund's adjuvant (complete for the initial injection, incomplete for booster injections) was subcutaneously administered into rabbits. Sera were collected after the fourth injection. Their specificity was tested by ELISA with the KLH peptide conjugate. Preimmune serum was collected before the first injection of antigen and unpurified sera were used in all cases. Polyclonal antisera used to detect SSIII, SSI, or SSIIa have been described previously (Cao *et al.*, 1999; Hennen-Bierwagen *et al.*, 2008).

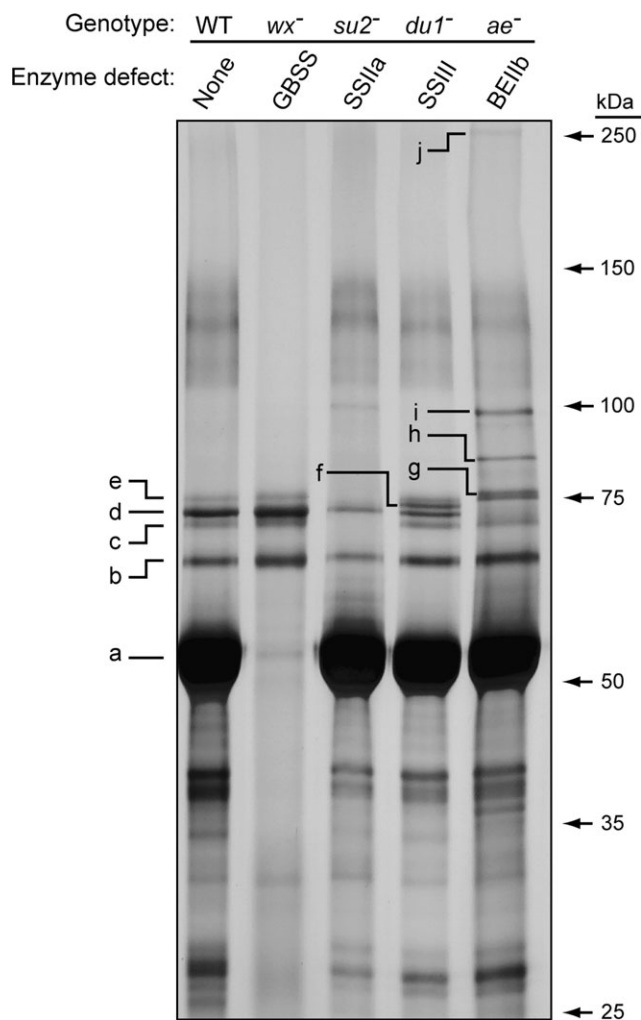
Proteins were electrophoretically transferred from SDS gels to nitrocellulose membranes in 0.1% SDS, 192 mM glycine, 25 mM TRIS, and 20% methanol. The membranes were soaked for at least 30 min in TTBS buffer (200 mM NaCl, 0.1% Tween 20, and 50 mM TRIS-HCl, pH 7.4) containing 5% Carnation instant milk to block non-specific binding sites. Polyclonal antisera or purified IgG fractions were then added and incubated 1 h with gentle shaking. The antibody probes and dilutions were as follows: αGBSS, 1/1000; αSSIg, 1/2000; αSSIIa, 1/1000; αSSIIHDP, 1/1000; αBEI, 1/2000; and αBEIIa/b, 1/5000. Following four washes with TTBS, blots were incubated with alkaline phosphatase-conjugated goat antirabbit IgG (Bio-Rad) at a 1:3000 dilution for 1 h. Blots were washed twice with TTBS and twice in TBS (200 mM NaCl and 50 mM TRIS-HCl, pH 7.4). Signals were visualized using the alkaline phosphatase conjugate substrate kit according to the manufacturer's instructions (Bio-Rad catalogue no. 170-6432).

## Results

### Internal granule-associated proteins in wild-type endosperm starch

Starch granules were isolated from developing endosperm of the non-mutant maize inbred W64A. To focus on internal granule-associated proteins, surface-associated proteins were removed by extensive washing in aqueous buffer containing SDS and β-mercaptoethanol. Proteins that remained tightly bound within the granule were isolated by gelatinization of the starch, separated by SDS-PAGE, and visualized by silver staining (Fig. 1). The staining pattern for the wild-type strain indicated a predominant protein of approximately 55 kDa (band a) and four clearly resolvable bands migrating between approximately 65 kDa and 75 kDa (bands b–e). Also present are a series of bands less than 50 kDa in molecular weight, as well as a group of diffuse signals located between the 100 kDa and 150 kDa molecular weight markers.

Each clearly resolvable protein from wild-type granules was identified directly by determining the amino acid sequence of proteolytic fragments. Proteins present in bands a–e isolated from standard inbred line W64A were digested with trypsin and the amino acid sequences of the



**Fig. 1.** Silver stain of internal starch granule-associated proteins. Proteins were separated by SDS-PAGE prior to staining. Molecular weight markers indicate the positions of known protein standards migrating in the same gel. The specific allele present in each mutant line is specified in the Materials and methods. Clearly resolvable bands are indicated by letters (a–i). The labelled bands are discussed in detail in the text, and the labels serve as references to further analyses, particularly mass spectrometry data.

resultant fragments were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Identifications were made based on a minimum of three peptide sequences perfectly matched with predictions from publicly available maize genomic or cDNA sequences (Table 2). All five labelled wild-type proteins were identified, as follows: band a, GBSS; band b, SSI; band c and band e, SSIIa; and band d, BEIIb (Table 2). Each of these proteins had previously been detected in maize starch granules by immunological- or direct sequencing methods (Table 1).

Immunological characterization using specific antisera confirmed the assignments made by amino acid sequence determination. Signals were detected for GBSS, SSI, SSIIa, and BEIIb (Fig. 2). The immunoblot data corresponded precisely with the mass spectrometry data, i.e.

band a was identified immunologically as GBSS, band b as SSI, band d as BEIIb, and band e as SSIIa. Band c, which was identified by peptide sequencing as SSIIa, did not react with any of the antisera (Fig. 2). In addition to the proteins observed in the silver stain, the immunoblot analyses also detected SSIII and trace amounts of BEI and BEIIa in wild-type granules (Fig. 2), even though the quantities of these proteins were below the level that could be detected by silver stain (Fig. 1).

The apparent molecular weight of GBSS, SSI, SSIIa, and BEIIa matches the predicted value of the cDNA sequences coding for the proteins (Table 2). In the case of BEI and BEIIa, the observed  $M_r$  is less than the predicted value of the cDNA sequences whereas the signal for SSIII is larger (Table 2). Anomalous migration in SDS-PAGE has been observed repeatedly in previous studies for various starch biosynthesis enzyme (Li *et al.*, 1999; Hennen-Bierwagen *et al.*, 2008).

#### Internal granule-associated proteins in mutant endosperm starches

Genetic analyses were used to verify the identity of the observed granule-bound proteins further, and also to test for pleiotropic effects that might indicate interactions among them. Internal granule-associated proteins were analysed from plants in the W64A genetic background homozygous for a mutation in either *wx*, *su2*, *ae*, or *du1*. These four genes code for GBSS (Klößgen *et al.*, 1986), SSIIa (Zhang *et al.*, 2004), BEIIb (Stinard *et al.*, 1993), or SSIII (Gao *et al.*, 1998), respectively.

Starch granules from the *wx*<sup>-</sup> mutant contained only the four high molecular weight proteins, bands b–e (Fig. 1). The wild-type appearance of these bands indicates that loss of GBSS does not affect association of any other granule-bound protein, specifically SSI, the two forms of SSIIa, or BEIIb (Fig. 1). The major 55 kDa protein identified as GBSS was absent from the bulk protein staining pattern for this mutant (Fig. 1) and no signal was detected in the immunoblot analysis using  $\alpha$ GBSS as the probe (Fig. 2). In addition, all but one of the lower molecular mass bands in the range of approximately 25–45 kDa were also absent from the bulk protein stain of the *wx*<sup>-</sup> mutant. These polypeptides, therefore, are likely to be proteolytic cleavage products of GBSS. To verify their identity further, two-dimensional electrophoresis was used to separate and analyse these lower molecular mass polypeptides (data not shown). LC-MS/MS analysis of the tryptic peptides derived from 18 spots distributed between the mature GBSS (55 kDa) and the bottom of the gel (~25 kDa) directly identified these fragments as portions of GBSS (data not shown). Another effect of the *wx*<sup>-</sup> mutation was elimination of the diffuse bands between 100 kDa and 150 kDa (Fig. 1), again suggesting that these bands are related to GBSS.

**Table 2.** Identification of internal granule-associated proteins by mass spectrometry sequencing

Band	Identified protein	Database accession no	No. of matched peptides	Predicted $M_r^a$ (kDa)	Observed $M_r^b$ (kDa)
a	GBSS	P04713	41	58	55
b	SSI	O49064	8	64	66
c	SSIIa	O48899	8	75	72
d	BEIIb	O81387	23	85	73
e	SSIIa	O48899	8	75	75
f	BEIIb	O81387	6	85	74
g	BEI	Q41740	5	86	76
h	BEIIa	O24421	3	91	90
i	$\alpha$ -(1 $\rightarrow$ 4)-glucan phosphorylase	TA188381_4577 <sup>c</sup>	8	n.a.	100
j	SSIII	O64923	3	188	250

<sup>a</sup> Predicted  $M_r$  values of mature proteins (i.e. after removal of plastid targeting sequences) were calculated from cDNA sequences originally published in the following references: SSIIa (Harn *et al.*, 1998); SSIII (Gao *et al.*, 1998); BEIIa (Gao *et al.*, 1997); BEIIb (Fisher *et al.*, 1993); SSI (Harn *et al.*, 1998); BEI (Baba *et al.*, 1991). The notation 'n.a.' indicates that full-length sequence data are not yet available.

<sup>b</sup> Observed  $M_r$  values were estimated based on comparison of electrophoretic mobility to that of known standard proteins.

<sup>c</sup> Translation of this assembled EST sequence from the Plant Transcript Assemblies (TaGI) databank (<http://plantta.tigr.org/index.shtml>) yields a sequence 88% identical to rice database entry Q9ATK9.

Confirming this conclusion, proteins in those diffuse bands were submitted for LC-MS/MS analysis and GBSS peptides were identified (data not shown).

The *su2<sup>-</sup>* mutant lacking SSIIa contained bands a, b, and d, identified as GBSS, SSI, and BEIIb, respectively. Bands c and band e were both missing from the bulk protein stain (Fig. 1), confirming their identification by LC-MS/MS as SSIIa. As expected, band e was missing from  $\alpha$ SSIIa immunoblot analysis of the *su2<sup>-</sup>* mutant (Fig. 2). The relative amounts of BEIIb and SSI present in the *su2<sup>-</sup>* mutant granules appear to be reduced, as indicated in both the immunoblot and the silver-stained gel (Figs 1, 2).

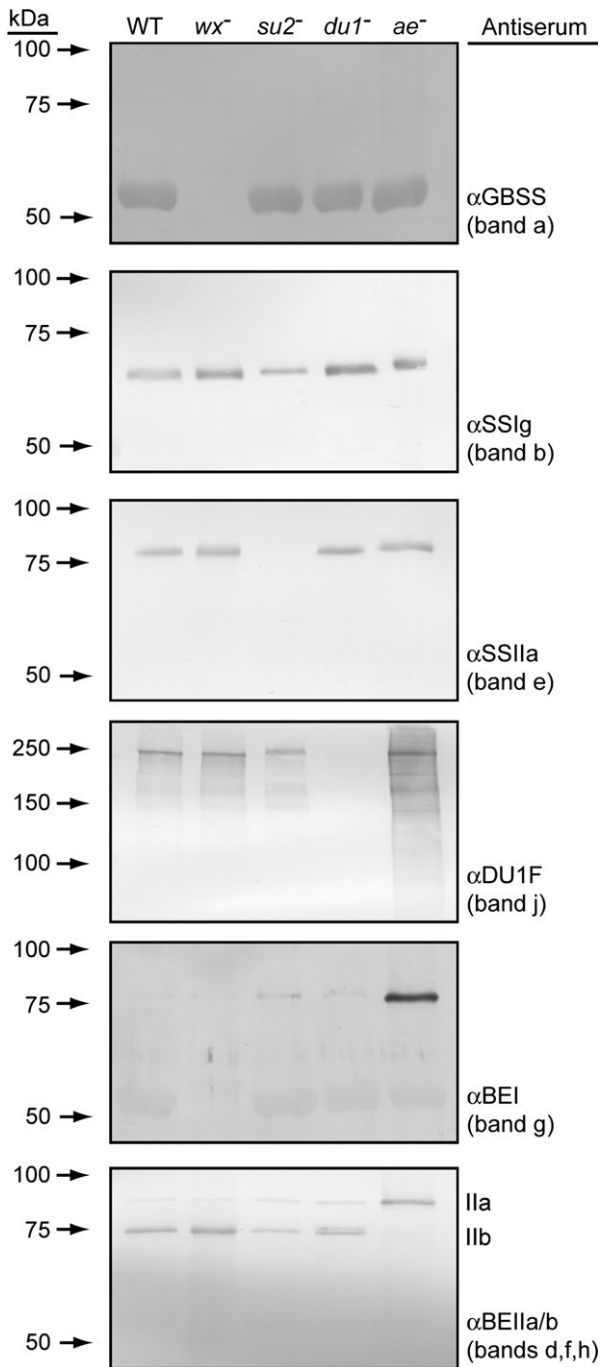
Loss of SSIII owing to the *dul<sup>-</sup>* mutation did not result in loss from the granules of any of the proteins present in the wild type, specifically GBSS, SSI, both forms of SSIIa, or BEIIb. However, a novel protein not observed in the wild type was present in the *dul<sup>-</sup>* mutant granules, designated as band f (Fig. 1). Mass spectrometry analysis identified this protein as BEIIb (Table 2), and the additional BEIIb band was also detected immunologically in the *dul<sup>-</sup>* mutant (Fig. 2). The level of the presumed wild-type BEIIb (band d) in *dul<sup>-</sup>* mutant granules is reduced compared to wild type, so it does not appear as if more of the enzyme associates with the granules (Fig. 1). Rather, loss of SSIII appears to result in an altered state of a subset of the total BEIIb molecules present in the granules.

The *ae<sup>-</sup>* mutation, in the gene coding for BEIIb, caused several changes in the complement of starch granule-bound proteins. As expected, the band previously identified as BEIIb (band d) was not detected in the mutant either in the bulk protein stain (Fig. 1) or the  $\alpha$ BEIIa/b immunoblot (Fig. 2). In addition, four proteins not observed by silver staining in wild-type granules were clearly detected in the *ae<sup>-</sup>* mutant, designated as bands g–j (Fig. 1). These proteins were excised from the gel and

subjected to peptide sequencing using LC-MS/MS. Three of the proteins were definitively identified by the amino acid sequences of tryptic fragments, as follows: band g, BEI; band h, BEIIa; band j, SSIII (Table 2). The fourth protein detected in the *ae<sup>-</sup>* mutant (band i), generated peptide sequences that did not match any annotated maize proteins in the current public databases. The fragments, however, exhibited perfect matches to the translation of a non-annotated EST sequence assembly present in the TIGR Plant Transcript Assemblies database (Table 2). Translation of that sequence and comparison to known protein sequences revealed 88% identity over 686 aligned residues to  $\alpha$ -(1  $\rightarrow$  4) glucan phosphorylase L isozyme, i.e. starch phosphorylase, from rice (Table 2). These data conclusively identify the 100 kDa protein from *ae<sup>-</sup>* mutant starch granules as starch phosphorylase (Genbank accession number EU684240). The same protein is also likely to be present in *su2<sup>-</sup>* mutant granules based on observation of a silver-stained protein of the same molecular weight (Fig. 1). The 100 kDa protein from the *su2<sup>-</sup>* mutant, however, was not characterized directly by LC-MS/MS.

Immunoblot analyses using specific antisera identified BEI, BEIIa, and SSIII as bands g, h, and j, respectively (Fig. 2), confirming the assignments from LC-MS/MS data. The immunoblots revealed that the granule-association of BEI, BEIIa, and SSIII is not specific to the *ae<sup>-</sup>* mutant, because they were also detected in the wild type, *su2<sup>-</sup>*, *wx<sup>-</sup>*, and for the two BEs in *dul<sup>-</sup>*. The quantity of BEI and BEIIa, however, increases to a large extent in the mutant lacking BEIIb compared with any of the other strains, and an apparent slight increase in the amount of SSIII associated with the granules was also evident.

The effects of each of the three mutations on internal granule-associated proteins were examined in independent biological replicates, i.e. in starch granules isolated from



**Fig. 2.** Immunoblot analysis of internal starch granule-associated proteins. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and hybridized with antiserum specific for GBSS, SSI, SSIIa, SSIII, BEI, or BEIIa and BEIIb. The position of molecular weight marker proteins run in the same gel are indicated.

different growth years and locations. In all instances the particular complement of proteins observed by SDS-PAGE and silver staining was reproducible for each genotype (data not shown). These results prove that the different protein profiles arise specifically from loss of the particular factor affected by each mutation, and rule out

the trivial explanation that the extent to which any protein associates with granules varies depending on growth conditions.

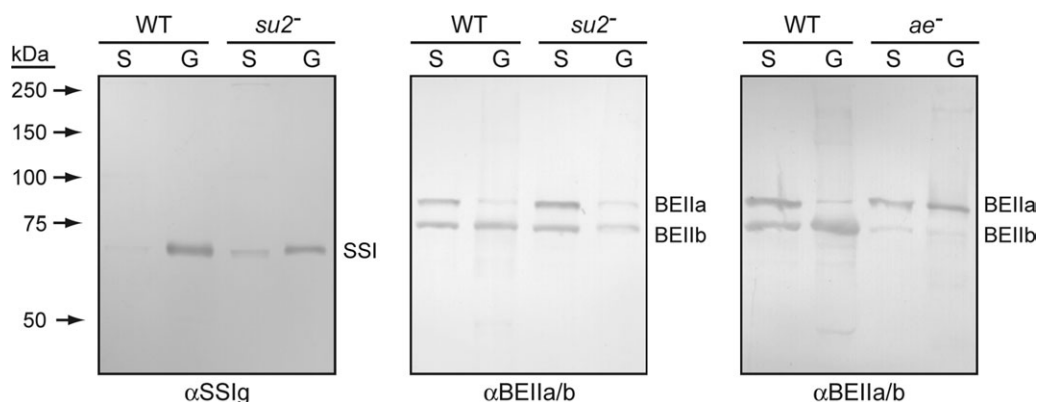
#### *Comparison of soluble and granule-bound proteins in wild-type and mutant granules*

The reduction in SSI and BEIIb levels in the granule as a consequence of the mutation eliminating SSIIa could be explained by overall gene expression level changes or by a specific reduction in granule association. In order to define the effects of the *su2*<sup>-</sup> mutation further, soluble extracts were prepared from amyloplast-enriched fractions from developing endosperm and starch-metabolizing enzymes were detected by immunoblot analyses (Fig. 3). The SSIIa-specific antiserum revealed a band of approximately 75 kDa in the wild-type amyloplast extracts, corresponding with the electrophoretic mobility of SSIIa known from the internal granule-associated protein analyses. This signal was totally absent in the *su2*<sup>-</sup> mutant extract (data not shown). The abundance of the approximately 66 kDa band detected by the SSI-specific antiserum was elevated in the *su2*<sup>-</sup> mutant amyloplast extract compared to wild type. The fact that SSI abundance in the soluble phase increases, at the same time that the level of this protein in the granules decreases, indicates that shifts have occurred in the relative distribution of the enzyme rather than changes in total protein content as a consequence of the *su2*<sup>-</sup> mutation. The amount of internal granule-associated BEIIb is also reduced in the SSII mutant, however, in this instance there was no obvious change in the level of the enzyme in the soluble amyloplast fraction (Fig. 3).

The same analysis was applied to the *ae*<sup>-</sup> mutant to test whether the major increase in the abundance of BEIIa in the granules as compared to the wild type resulted from a redistribution of the protein between the granule and stromal fractions. The results showed that as the amount of BEIIa in the granules increases there is a decrease in the abundance of the protein in the soluble phase (Fig. 3). Thus, redistribution between granule and stroma appears to be the explanation for altered abundance of internal granule-associated proteins, as opposed to changes in the total level of the protein present in the cell.

#### *Phosphorylation of internal starch granule-associated proteins*

Phosphorylation of BEIIb, SSIIa, and starch phosphorylase in wheat amyloplast soluble extracts has been detected previously by radioactive labelling using  $\gamma$ -<sup>32</sup>P-ATP (Tetlow *et al.*, 2004b). Staining with the phosphoprotein-specific dye ProQ Diamond (Steinberg *et al.*, 2003) was used to examine whether the internal granule-associated proteins from maize endosperm are similarly modified. Specificity of the stain was demonstrated using



**Fig. 3.** Soluble-granule distribution of SSI, BEIIa, and BEIIb. Internal granule-associated proteins (G) or soluble amyloplast lysates (S) from wild type, a *su2<sup>-</sup>* mutant, or an *ae<sup>-</sup>* mutant were separated by SDS-PAGE. Each lane was loaded with 15  $\mu$ g of proteins. Gels were analysed by immunoblotting using the indicated antiserum. The positions of known molecular weight standards migrating in the same gel are indicated.

two different sets of standards containing a mixture of both phosphorylated and non-phosphorylated proteins, and in both instances only the phosphorylated proteins were detected (Fig. 4).

Three clearly identified polypeptide bands exhibited a positive phosphorylation signal, corresponding to GBSS, BEIIb, and starch phosphorylase (Fig. 4). The identity of phosphorylated GBSS and BEIIb was demonstrated both by electrophoretic mobility comparison to the sequenced proteins and by absence of the signal from the *wx<sup>-</sup>* mutant or the *ae<sup>-</sup>* mutant, respectively (bands a and d in Figs 1 and 4). The distinct electrophoretic mobility of starch phosphorylase and its abundance in the *ae<sup>-</sup>* mutant (band i in Figs 1 and 4) served to identify the phosphorylated form of that protein.

Phosphorylation of GBSS appears to be affected by mutations in other starch biosynthetic enzymes, as judged by the intensity of the ProQ Diamond stain. Specifically, the signal is stronger in the line lacking SSIIa and weaker and more diffuse in the line lacking BEIIb. This observation suggests that alterations either in starch structure and/or interactions between biosynthetic enzymes can affect phosphorylation.

## Discussion

### Identification of internal granule-associated proteins

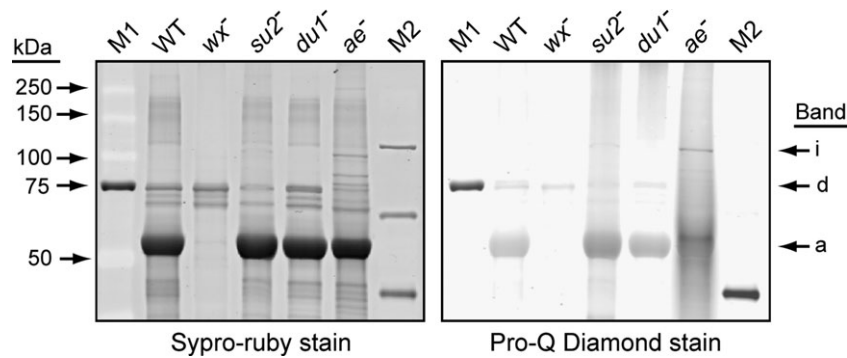
The overall objective addressed in this study is to identify all proteins located in the interior of starch granules from maize endosperm, and also to characterize the post-translational modifications that occur on these proteins. Experimentally, the internal granule-associated proteins are defined by resistance to protease and/or detergents applied to intact granules, and release by solubilization of the granule polymers. The results presented here confirmed previous findings of GBSS, SSIIa, SSI, and BEIIb as internal granule-associated proteins that are readily

detected by mass staining (Table 1). In addition, SSIII, BEI, BEIIa, and starch phosphorylase were also found as internal granule-associated proteins. Thus, of the five conserved SSs present in maize, four of them are located within starch granules. The fifth conserved class of this enzyme, SSIV, is either not present in granules or is there in extremely low levels compared with the other four classes. Similarly, all three of the conserved forms of BE present in maize are found in the interior of starch granules.

The proteins not found in previous studies as internal granule-associated proteins, specifically SSIII, BEI, and BEIIa were detected in the granules using new antisera or IgG fractions that were not available previously. Also, these three proteins and starch phosphorylase were readily detectable at the level observed by LC-MS/MS in granules of the *ae<sup>-</sup>* mutant lacking functional BEIIb (Fig. 1). This observation coupled with immunoblot analysis (Fig. 2) revealed that SSIII, BEI, and BEIIa are present in wild-type granules as well, apparently at levels significantly below those of SSI, SSIIa, and BEIIb. This point is emphasized by the bulk protein stain in which SSIII, BEI, and BEIIa cannot be observed in the wild-type granules (Fig. 1).

GBSS was identified as the super-abundant protein of molecular mass  $\sim$ 55 kDa, as expected from the knowledge that this enzyme typically accounts for about 85% of total internal granule-associated protein (Echt and Schwartz, 1981). Multiple polypeptides of relatively small molecular mass, approximately 25–50 kDa were also identified directly by amino acid sequence as fragments of GBSS. These polypeptides have been observed previously in maize (Mu-Forster *et al.*, 1996) and wheat (Rahman *et al.*, 1995), and more recent two-dimensional PAGE analyses of barley endosperm proteins distributed between 60 kDa and  $\sim$ 25 kDa suggests they are N- or C-terminal truncations of mature GBSS (Boren *et al.*, 2004). The banding pattern of the GBSS fragments is





**Fig. 4.** Phosphorylation-specific stain of internal starch granule-associated proteins. Proteins from the indicated mutants were separated by SDS-PAGE and the same gel was stained sequentially with Pro-Q Diamond to reveal phosphoproteins and then with Sypro Ruby to reveal total protein. The position of known standards migrating in the same gel is indicated. Mixtures of phosphorylated- and non-phosphorylated control proteins were used as internal controls for detection of phosphoproteins by Pro-Q Diamond (M1 and M2).

reproducible, rather than an apparently random distribution of molecular weights, which suggests that cleavage of the protein occurs *in vivo* in the course of starch granule synthesis.

Maize mutations in the genes coding for GBSS, SSIIa, SSIII, or BEIIb were used to verify the identity of each protein band. In each instance the signal of the expected molecular weight was missing, thus confirming the identification. In the GBSS mutant a sharp band of approximately 55 kDa is observed, which normally is hidden beneath the super-abundant protein band of the same molecular weight (Fig. 1). The identity of this protein remains to be determined, however, it is most unlikely to be related to GBSS because it is not detected by  $\alpha$ GBSS antiserum (Fig. 2).

SSIIa was detected as two electrophoretic mobility forms by amino acid sequence determination and, as expected, both of those bands were absent from the *su2<sup>-</sup>* line carrying a null mutation in the gene coding for SSIIa. Immunoblot analysis using  $\alpha$ SSIIa revealed only one of these two bands, but at present the reason for this specificity cannot be determined. A possible explanation for the two forms of SSIIa is that the protein is differentially post-translationally modified and this changes an important antigenic site so that the signal for the faster migrating protein is lost. Two SSIIa mobility forms were previously suggested by analyses of starch granules in maize (Zhang *et al.*, 2004) and rice (Umemoto and Aoki, 2005) in which presumably specific immunoblot analyses identified two bands. By contrast, available data from other species, specifically potato, pea, or barley, indicate only a single form of SSIIa (Table 1).

Finally,  $\alpha$ -(1  $\rightarrow$ 4) glucan phosphorylase (starch phosphorylase) was also identified as an internal starch granule-associated protein in the *ae<sup>-</sup>* mutant. Starch phosphorylases catalyse the reversible transfer of a glucosyl unit from the non-reducing end of an  $\alpha$ -(1  $\rightarrow$ 4) glucan chain to inorganic phosphate ( $P_i$ ) to produce glucose-

1-phosphate (G1P). Low G1P concentration and a high  $P_i$ /G1P ratio in the amyloplast indicate that the enzyme does not have a starch biosynthetic function (Mu *et al.*, 2001; Smith *et al.*, 1997). Consistent with this view, analysis of leaves from mutants of potato (Sonnewald *et al.*, 1995) or *Arabidopsis* (Zeeman *et al.*, 2004) lacking starch phosphorylase have not revealed any impact on starch metabolism. Other genetic evidence, however, provides support for a role of this enzyme in starch biosynthesis. For example, plastidial starch phosphorylase activity coincides with starch accumulation in developing wheat endosperm (Schupp and Ziegler, 2004). In maize, at the same time, *shrunk4* mutants display a strong reduction in the amounts of starch and a significant modification of the electrophoretic migration pattern of starch phosphorylases (Tsai and Nelson, 1969). Genetic analyses in *Chlamydomonas* showing that mutation of a plastidial starch phosphorylase causes reduced starch accumulation also support a biosynthetic role (Dauvillee *et al.*, 2006). Finally, although a single mutation of the plastidial phosphorylase (PHS1) has no significant influence on starch structure and accumulation in *Arabidopsis* leaves, a double mutation of both phosphorylase and SSIV has a significant influence on granule size (Planchot *et al.*, Patent EP1882742). This point supports the suggestion that PHS1 could be involved in complexes of functional importance for granule formation, and the finding that starch phosphorylase associates with BEI and BEIIb in a complex in wheat endosperm supports this hypothesis (Tetlow *et al.*, 2004b). The present study, which identifies starch phosphorylase as an internal granule-associated protein in the *ae<sup>-</sup>* mutant, is consistent with a biosynthetic role.

#### *Pleiotropic effects of mutations eliminating specific internal granule-associated proteins*

Three of the maize mutants analysed in this study exhibited altered binding of more than one protein to the

granule, affecting proteins other than the product of the mutant gene. In the *su2<sup>-</sup>* mutant, loss of SSIIa also resulted in decreased association of SSI, BEIIb, and potentially SSIII with the starch granule (Figs 1, 2), and an increase in SSI levels in the amyloplast lysate (Fig. 3). This consequence is similar to reported effects of SSIIa deficiencies in wheat, barley, and rice (Morell *et al.*, 2003; Umemoto and Aoki, 2005; Yamamori *et al.*, 2000). In barley, the SSIIa deficiency also causes a major reduction in the amount of GBSS bound to the granules (Morell *et al.*, 2003), however, this effect was not observed in maize (Fig. 1), wheat (Yamamori *et al.*, 2000), and rice (Umemoto and Aoki, 2005). Possible explanations for this phenomenon proposed previously were either that SSIIa directly binds to other starch biosynthetic enzymes and thus affects their association with granules, or that loss of SSIIa changes starch granule structure such that general or specific protein binding is reduced (Morell *et al.*, 2003). The former hypothesis is consistent with recent findings that in amyloplast lysate from wheat or maize SSIIa is present in complex(es) that also contain SSI, BEIIa, BEIIb, and/or SSIII (Hennen-Bierwagen *et al.*, 2008; Tetlow *et al.*, 2008).

Internal granule-associated proteins from the *dul<sup>-</sup>* mutant lacking SSIII included an additional form of BEIIb, distinguishable by its altered migration to BEIIb in wild-type, *su2*, and *wx<sup>-</sup>* mutant starch granules. Given that the migratory distance between the two forms is small, this novel BEIIb band may represent a post-translationally modified state of the protein that uniquely binds to *dul<sup>-</sup>* mutant granules. Consistent with this hypothesis, BEIIb stains with Pro-Q Diamond and is thus identified as a phosphorylated protein (Fig. 4). At this time, the reason that a particular form of BEIIb might be found associated with the mutant granules but not the wild starch cannot be discerned. The fact that SSIII binds multiple starch biosynthetic enzymes in amyloplast cell extracts is consistent with the suggestion that direct interactions between SSIII and BEIIb occur in the granule, and this could explain the difference in BEIIb state between the wild type and *dul<sup>-</sup>* mutant starch.

Finally, loss of BEIIb owing to the *ae<sup>-</sup>* mutation causes changes in the abundance of several different internal granule-associated proteins. As expected, BEIIb is missing from these granules (Figs 1, 2). In addition, four distinct proteins were observed by silver stain in *ae<sup>-</sup>* mutant granules that were not present in wild-type granules, specifically BEI, BEIIa, SSIII, and starch phosphorylase (Fig. 1, bands g–j). Loss of BEIIb evidently affects the degree of association of BEI, BEIIa, and SSIII with starch granules, rather than enabling that association. Immunoblot analyses revealed that BEI, BEIIa, and SSIII are also present in wild-type granules, although at a lower level than in *ae<sup>-</sup>* mutant granules (Fig. 2). Immunological characterization has not yet been performed for starch

phosphorylase and so, in this instance, it cannot yet be determined whether or not the enzyme is present at low levels in normal starch.

Again there are two possible explanations for differential association of specific biosynthetic enzymes with starch granules. Structural changes in amylopectin that occur as a result of loss of BEIIb could offer a more efficient glucan substrate for binding of BEI, BEIIa, starch phosphorylase, and SSIII. Alternatively, direct physical interactions between BEIIb and the other starch biosynthetic enzymes could contribute to their increased levels in *ae<sup>-</sup>* mutant granules. BEIIb was found in wheat amyloplasts in a precipitable complex along with BEI and starch phosphorylase (Tetlow *et al.*, 2004b), and in maize amyloplast extracts BEIIb was found to co-purify in a high molecular weight complex coincident with SSIII (Hennen-Bierwagen *et al.*, 2008). Although all of these interactions have been identified among soluble endosperm proteins, it is reasonable to infer that they also occur within the starch granule and that disruption of one or more complexes involving BEIIb could lead to altered binding and/or activities of the other enzymes. The overall three-dimensional structures of all three BE forms are certainly similar, so, potentially, BEIIb may compete with BEIIa and BEI for specific granule and/or protein binding sites. In this instance, loss of BEIIb in the *ae<sup>-</sup>* mutant could allow for increased binding of BEI and BEIIa to complexes that occur as internal granule-associated proteins.

#### *Phosphorylation of internal-granule associated proteins*

Staining the internal granule-associated proteins using a phospho-protein specific dye revealed phosphorylation of at least three proteins, GBSS, BEIIb, and starch phosphorylase. The result for BEIIb and starch phosphorylase is consistent with the previous finding that the soluble forms of BEIIb and starch phosphorylase in wheat amyloplasts accept transfer of a radioactive phosphate group from labelled ATP (Tetlow *et al.*, 2004b). In the same study, SSIIa was also found to be phosphorylated in wheat amyloplast extracts. The fact that SSIIa in maize starch granules did not stain with ProQ-Diamond does not rule out the possibility that it is also phosphorylated, considering the lower sensitivity of the dye method compared with that of radioactive labelling. Many questions regarding phosphorylation of internal granule-associated proteins remain to be addressed. These include the stoichiometry of phosphorylation, whether or not phosphorylation affects binding to glucans in the granule and/or to other internal granule-associated proteins, and whether or not enzyme activity is regulated by the post-translational modification.

## Supplementary data

Supplementary data can be found at *JXB* online.

**Fig. S1.** Identification by LC-MS/MS of specific peptides for SS and BE isoforms. LC-MS/MS data are shown for individual peptides originating from SSI (A), SSIIa (B), SSIII (C), BEI (D), BEIIa (E), or BEIIb (F).

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